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Stomatal Responses to Light and Drought Stress in Variegated Leaves of *Hedera helix*[1]

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ABSTRACT

Direct and indirect mechanisms underlying the light response of stomata were studied in variegated leaves of the juvenile phase of *Hedera helix* L. Dose response curves of leaf conductance were measured with blue and red light in leaves kept in normal or in an inverted position. In the green portions of the leaves, the sensitivity to blue light was nearly 100 times higher than that to red light. No response to red light was observed in the white portions of the leaves up to 90 micromoles per square meter per second. Red light indirectly affected leaf conductance while blue light had a direct effect. Leaf conductance was found to be more sensitive to drought stress and showed a more persistent aftereffect in the white portions of the leaves. A differential effect of drought stress on the responses to blue and red light was also observed.

The fundamental role played by light (14, 31) and plant water status (14) in controlling stomatal aperture in most plants is well documented. However, interactions of irradiance with leaf water status have not been adequately examined (6).

The effect of light on stomata can be defined as direct or indirect depending on the localization, in or outside the stomata, of the photoreceptor involved. The existence of both kinds of effects is generally accepted and it has been proposed that both blue and red light participate in direct effects through a blue light photoreceptor and Chl, in addition to an indirect response elicited by changes in the C_i[2] (31). The relative importance of these light responses is a matter of current interest (31). The existence of several mechanisms controlling stomatal movements might have ecological implications.

Variegated leaves make it possible to compare in the same organ the response of stomata produced by direct plus indirect effects (in green portions), with that produced by direct effects alone (in white portions). In this work the response of g_l to blue and red light and to water stress was compared using variegated leaves of *Hedera helix*. The interactions between these factors were also tested.

MATERIALS AND METHODS

Plant Culture. The plants of *Hedera helix* L. used in this study were in the juvenile phase. These were grown from cuttings in pots filled with a mixture of soil and sand and kept in a glasshouse. They were fertilized at monthly intervals with a commercial N-P-K fertilizer. The plants were transferred from the glasshouse to a growth chamber 7 d or more before the beginning of the experiments. This chamber was set to a constant temperature of 25 ± 1°C and a photoperiod of 12 h. The photosynthetically active photon flux supplied by nine fluoresent tubes (Philips TLF 40W/33) was 200 μmol m^{-2} s^{-1} at leaf level. Humidity was not controlled but vapor pressure deficit was always 6 mbar or less.

Measurements and Observations. Leaf conductance was measured with a diffusion porometer (Li-Cor LI-60 with a Lambda LI-20S sensor). Leaf water potential was measured with psychrometric chambers (Wescor C-51) and a microvoltmeter (Wescor MJ-55). Discs of 33.2 mm^2 were cut from the leaves with a paper punch. The chambers were kept inside an incubator at 25 ± 0.1°C; equilibration time was 4 h. Before the start of the experiment the chambers were calibrated at the same temperature using KCl solutions.

Chl content was measured in 90% acetone extracts (23) with a Metrolab 2500 double beam spectrophotometer using equations derived by Beale (3). The transmittance spectra of the green and white parts of the leaves were measured with a spectroradiometer (ISCO model SR). The fluorescence of the abaxial epidermis was observed with a Leitz microscope, using paradermal sections of *H. helix* leaves mounted in water between a slide and coverslip. A BG12 exciter filter and a K510 barrier filter were used. Stomatal frequency and dimensions were measured from cyanoacrylate cement imprints (29).

Plants were irradiated in an air-conditioned darkroom. Red light was obtained from unfiltered Philips fluorescent light tubes TL-40W/15, and blue light from Philips mercury vapor lamps HP 400W, filtered through 4 cm of water and two layers of blue Plexiglas each 3 mm thick (B-27, Rohm and Haas, Darmstadt, Germany). The spectral energy distribution of both light sources was measured with a spectroradiometer (ISCO model SR) (Fig. 1). Irradiance was measured with a Kipp solarimeter. All light measurements were transformed to quantum flux.

Experimental Procedure. Three different experimental protocols were used: (a) light treatments in unstressed plants; (b) water stress treatments under saturating white light; and (c) light treatments in stressed plants.

In the first protocol, leaves in a normal or an inverted position were irradiated with either blue or red light throughout a day. Light intensities were increased and never decreased throughout the day so as to avoid possible errors caused by hysteresis in the response. Only plants with closed stomata ($g_l < 0.3$ mm s^{-1}) at the beginning of each day, after pretreatment of at least 14 h in darkness, were used. Measurements were not taken during the first 2 or the last 3 h of the photoperiod. Temperature was kept within 25 ± 3°C during irradiation. The plants were kept for 2 h at constant irradiance before measuring g_l (previous tests indi-

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[2] Abbreviations: g_l, leaf conductance; ψ_l, leaf water potential; PFD, photon flux density; HMR, half maximum response; C_i, intercellular CO_2 concentration; b/r, ratio between blue and red light PFDs.

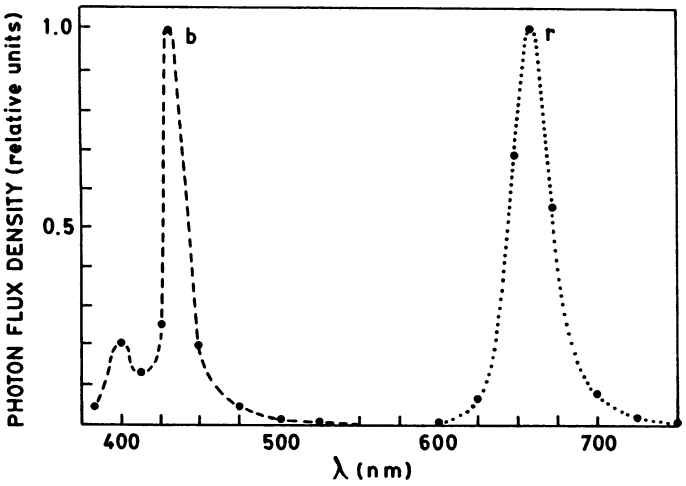

FIG. 1. Quantum emission spectra of blue (b) and red (r) light sources.

cated that this time interval was sufficient to obtain steady state g_l values). No safelights were used; the dark g_l values were taken at the beginning of irradiation with low intensity blue or red light.

In the second protocol, water stress was imposed by withholding water until stomatal closure ($g_l < 0.3$ mm s^{-1}) in white and green portions of the leaves. The g_l was measured under saturating white light (200 μmol m^{-2} s^{-1}) between the 2nd and 3rd h of the photoperiod. Water potentials were measured in replicate experiments.

In the last protocol, water stress was imposed as in the preceding experiment, but each day g_l was measured under a PFD of 1 μmol m^{-2} s^{-1} blue and 85 μmol m^{-2} s^{-1} red light (calculated according to the data of Figure 3 to give a g_l equal to 2 mm s^{-1}, slightly less than HMR), and under saturating white light (200 μmol m^{-2} s^{-1}). The sequence of irradiation was: 2 h under white light, 1.5 h in darkness, 2 h under blue, 1.5 h in darkness, 2 h under red, and then under white light until the end of the photoperiod. For half the plants the order of blue and red irradiation was inverted, the plants being assigned randomly each day to the two groups. Leaf conductance was measured at the end of each of the light and dark periods.

Statistical Analysis. Leaf conductance means were compared by use of *F*-test in an ANCOVA (27). Red and blue light treatments were analyzed separately and means were adjusted to mean irradiances (46.0 and 1.36 μmol m^{-2} s^{-1}, respectively). Regression lines were fitted to treatments and the homogeneity of slopes within each light quality was tested with an *F*-test in an ANCOVA, and between blue and red light treatments with a *t*-test for two independent regressions (27). The results of the second experimental protocol were analyzed by fitting functions, using least squares, to data from each experimental unit, obtained before or after rewatering; and the parameters of interest were then tested by ANOVA (30). The functions used are piecewise-linear in the limit (Ref. 20, equations 3.1 and 3.5). These functions were selected because they provide parameters that can be interpreted as the time of transition between the plateau and the sloping segment of the curve and the g_l at the plateau. The difference between blue and red light treatments in stressed plants was tested by comparing residuals after fitting different regression models. In this experiment a crossover design was used for light quality, so error terms are different for this factor and water stress.

RESULTS

Hedera helix leaves are hypostomatous. Neither stomatal density (mean of 150 stomata mm^{-2}) nor pore length were different in white and green portions of the leaves. White portions were thinner than the green ones (170 and 210 μm thick). The latter had two layers of distinctly elongated palisade parenchyma cells, while in the white portions all mesophyll cells were nearly isodiametrical. The characteristics observed in the green portions agree with those reported by Bauer and Bauer (2) for leaves of the juvenile phase of this species.

The transmittance of the white portions of the leaves was much higher than that of the green portions, but a small trough in the red region of the spectrum was detected (Fig. 2). Differences in transmittance due to exposing the abaxial or adaxial faces of the leaves to the incident beam were minimal. Although the white portions were not free of Chl, the Chl content was much lower than in the green portions: 2.49 mg m^{-2} and 259 mg m^{-2}, respectively. Fluorescence microscopy observations using dark field illumination revealed chloroplasts in guard cells and epidermal cells, in both green and white parts of the leaves. Epifluorescence observations of *Hedera* variegated leaves agree with our results, and also show small patches of residual mesophyll fluorescence in the white portions (E Zeiger, personal communication).

Effect of Light in Unstressed Plants. The relation between the logarithm of PFD and g_l was linear, and the slope did not differ significantly ($P > 0.10$) between treatments (Fig. 3). The mean g_l response to blue light (adjusted to equal PFD) was different in white and green portions of the leaves, and the effect of leaf inversion was significant only in the green portions. White portions in either normal or an inverted position and green portions in a normal position required 1.7 μmol m^{-2} s^{-1} for HMR (as measured in the same leaves under white light) and inverted green portions required only 0.34 μmol m^{-2} s^{-1}. Stomata did not open in the white portions with PFD of red light up to 90 μmol m^{-2} s^{-1}. In the green portions HMR in red required 110 μmol m^{-2} s^{-1}, and leaf inversion had no effect.

The photon flux density required at the abaxial epidermis for a g_l equal to HMR of this surface was used as a measure of sensitivity to light in comparisons between species. Under blue light, in *H. helix* it was 0.1 times of that required in *Xanthium strumarium* (Fig. 1 in Ref. 24) and only 0.01 times of that required in *Zea mays* (Fig. 1 in Ref. 22). Under red light in *H. helix* it was between 3.5 and 4 times that required in *X. strumarium* (Fig. 1 in Ref. 24) and *Z. mays* (Fig. 1 in Ref. 22).

Effect of Water Stress under Saturating Irradiance. In agreement with observations in many species (14) drought stress caused a severe reduction in g_l, but green and white portions responded differently. Withholding water caused an earlier reduction of g_l in the white portions of the leaves, and on rewater-

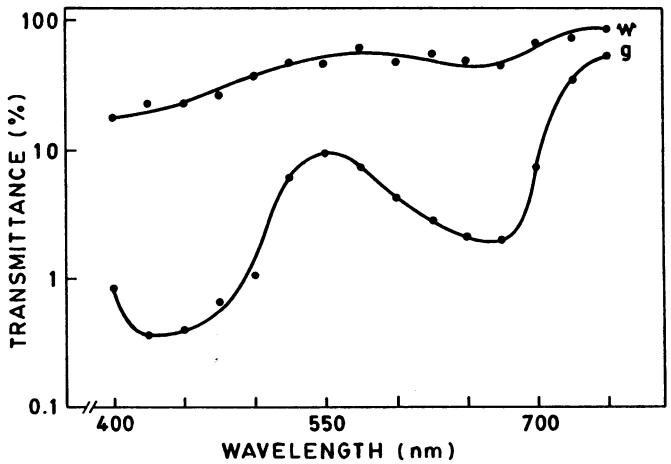

FIG. 2. Transmittance spectra of white (w) and green (g) portions of a variegated leaf of *H. helix*.

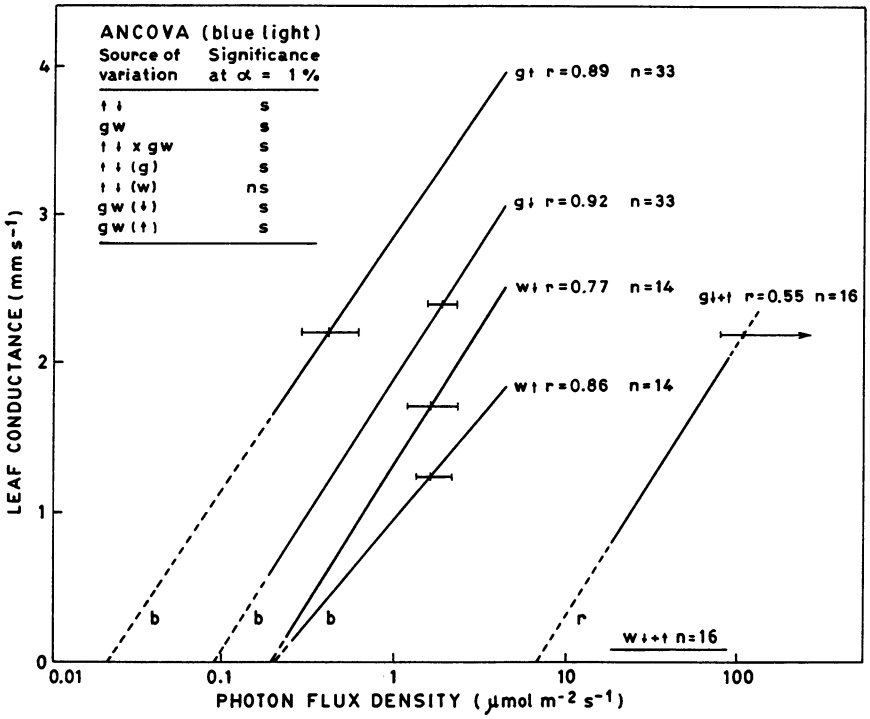

FIG. 3. Relation between leaf conductance and PFD of blue (b) and red (r) light, in white (w) and green (g) portions of variegated leaves, in normal (↓) or inverted (↑) position. Confidence intervals, at P = 0.05, for the PFD required for HMR are indicated by horizontal bars. Dashed lines are extrapolated.

ing, the aftereffect of stress also persisted longer in these portions (Fig. 4). The recovery after rewatering was complete in both white and green portions (Fig. 4). The decrease of g_l in the white portions started at about the same time as the decrease of ψ_l and in the green portions between 2 and 3 d later (Fig. 5). The highest value of ψ_l was observed the day after rewatering, that is, before g_l recovered completely. Hence, the effect of water stress on g_l persisted after ψ_l returned to the control values. No differences were observed between the ψ_l values of white and green portions of the same leaves (Fig. 5, and measurements with leaf psychrometers under slightly different environmental conditions (Data not included).

Effect of Light Quality in Drought Stressed Plants. This experiment was designed to detect possible interactions between the effects of light quality and drought stress. Water was withheld from the treated plants and the responses of green portions to blue and red light were tested while g_l responded to drought stress and to rewatering. Blue and red light PFD were adjusted so as to have similar relative g_l in the controls (*ca.* HMR). Leaf conductances after rewatering were significantly higher under red than blue light, when analyzed for the whole 5 d period (Fig. 6A). The blue light response had a somewhat longer lag before it began to rise after recovery of ψ_l. However the difference was small and decreased as the response in both spectral regions approached the control values (Fig. 6A). Under saturating white light, the aftereffect disappeared earlier than under nonsaturating blue or red treatments (Fig. 6B).

DISCUSSION

Effect of Light. In white portions of unstressed leaves maximum g_l values measured under saturating white light were

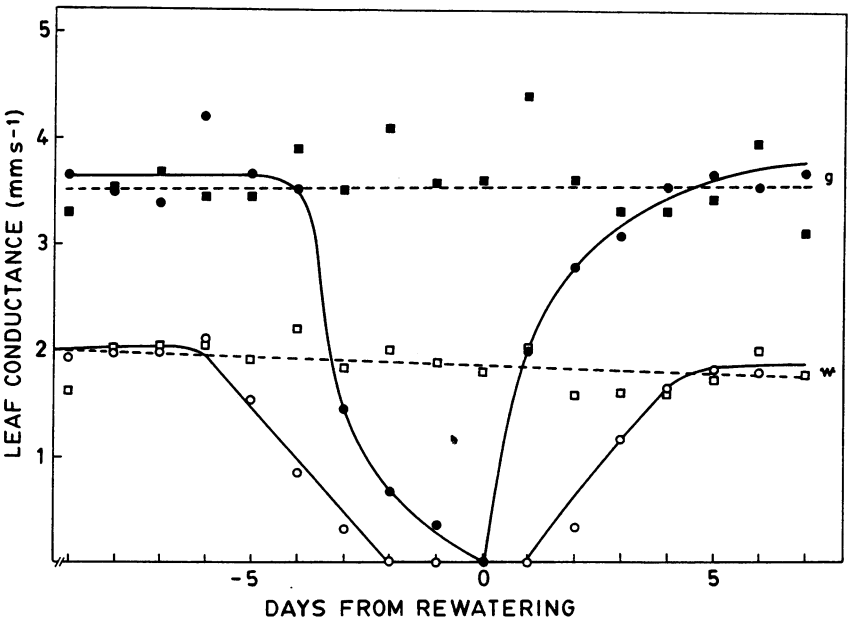

FIG. 4. Relation between leaf conductance and time, in white (w) and green (g) portions of variegated leaves; stressed plants (——) were not watered from d −10 to d 0; controls (- - - - -) were watered every other day. Leaf conductance was measured between the 2nd and 3rd h of the photoperiod. The SE for maximum g_l, before and after stress, was 0.15 mm s^{-1} ($df = 9$) for both green and white portions. Leaf conductance started to decline in white and green portions, 6.7 and 3.9 d before rewatering (SE = 0.06, $df = 6$) and recovered in 5.0 and 1.6 days (SE = 0.10, $df = 6$).

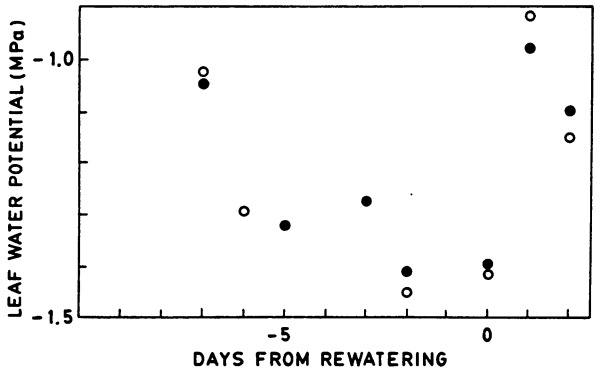

FIG. 5. Relation between leaf water potential and time, in white (O) and green (●) portions of variegated leaves of stressed plants, that were not watered from d −9 to d 0. Water potential was measured between the 3rd and 4th h of the photoperiod.

approximately half of those in the green portions (Fig. 4). Normal light responses of white parts of leaves have been reported in some species (1, 10); other studies reported lack of response or sluggish movements (28). The difference in g_l observed in *H. helix* between white and green portions could not be explained either by differences in the stomatal frequency nor in pore length, hence conductance differences must be related to stomatal aperture.

In juvenile leaves of *H. helix* Bauer and Bauer (2) measured a light compensation point for photosynthesis of 1.8 μmol m^{-2} s^{-1} at 20°C. This irradiance is higher than that required for HMR using blue light in the green portions of variegated leaves, and slightly lower than the irradiance for $g_l = 0$, calculated by extrapolation of the red light dose-response line. That is, the response of the stomata of ivy to blue light starts well below the compensation point, and that to red light, above it. Moreover, under blue light, leaf inversion caused a displacement of the dose response line with no change in slope in the green portions, but not in the white portions. This would be expected if the photoreceptor was exposed to a higher PFD when not shaded by the mesophyll. These data are consistent with the hypothesis of the existence of two photoreceptor systems: one located in the mesophyll, dependent on the presence of Chl and sensitive to blue and red light of PFD high enough to significantly change C_i through photosynthesis; and another one located in the abaxial epidermis and highly sensitive to blue light. A more comprehensive model has been proposed by Brogardh (4) to explain light dependent stomatal movements in *Avena*. The data presented here are also consistent with this model, except for a slow response to blue light in *H. helix* (PJ Aphalo, RA Sánchez, unpublished data). In different studies the effect of leaf inversion on stomatal responses has been considered an indication of the location of the photoreceptors (22, 24). The lack of response to leaf inversion under red light (Fig. 3) is evidence of a null or at least small direct effect of red light in our experimental setting. There are at least three possible explanations for this: (a) the contribution of the direct Chl-dependent photosystem might be always small in *H. helix*, (b) this contribution could be important only at irradiances higher than those available from the red light source used in this work, or (c) a background irradiation with blue light could be necessary to elicit a direct response to red light, such an effect of blue light on malate formation has been observed in *Vicia faba* guard cells (17).

Dose response lines differ between white and green portions only in their x intercept when measured under low irradiance blue light (close to or below the compensation point), its value being higher in the white parts (Fig. 3). Although the existence of an interaction between the direct response to blue light and C_i cannot be ruled out, the difference we measured indicates a change in the perception of the light signal by the leaf. The white parts of the leaves of *H. helix* have apparently functional chloroplasts in the guard cells, and only small residual patches of Chl containing mesophyll cells. Nevertheless, the lack of response to red light and the existence of a response to low irradiance blue light (Fig. 3) is similar to what has been observed in a Chl deficient barley mutant (26) and in wheat treated with the herbicide SAN 9789 (9). This information shows that photosynthesis is not always necessary for light induced stomatal aperture. It also gives further evidence of the lack of a Chl dependent direct effect in *H. helix*.

Most attempts to assess 'direct' and 'indirect' effects of light on stomata of different species have utilized gas exchange experiments in which 'direct' mean independent of the estimated C_i or where photosynthesis was halted by means of inhibitors (25). A different approach to this problem is to make comparisons based on the spectral response. The ratio between the quantum flux densities of blue and red light that would give the same degree of opening (b/r) is a good measure for establishing differences when comparing stomatal responses of different species (13). On the assumption that there are only two photosystems involved, that they have different relative activities in the blue and red regions of the spectrum, and that there is no effect of screening by other pigments, this ratio would give a measure of the participation of each in the observed stomatal response. The maximum possible values of b/r would be that of photosynthesis, and the minimum that of the blue absorbing photosystem. From published dose response data it is possible to calculate the b/r ratio with the irradiances required to obtain HMR. This ratio is almost always smaller than that of photosynthesis, varying widely between species, and even between sun and shade leaves of the same plant (Table I). This great variability is in itself an indication that the photosystems involved in stomatal responses to light are

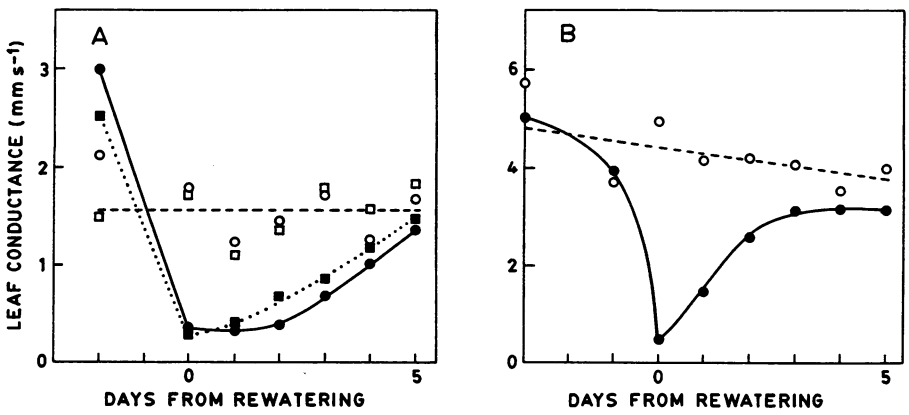

FIG. 6. Relation between leaf conductance of green portions of variegated leaves, and time: A, under blue (O, ●) and red (□, ■) light PFDS, calculated to give a g_l equal to 2 mm s^{-1} in unstressed plants (1 and 85 μmol^{-2} s^{-1}, respectively); and B, under saturating white light (200 μmol^{-2} s^{-1}). Stressed plants (●) were not watered from d −4 to d 0, controls (O) were watered every other day. The functions fitted to each of the three light treatments in stressed plants gave $r^2 > 0.99$; for blue and red light treatment g_l maximum was assumed to be equal to the controls. The time for recovery was with white, red, and blue light: 2.24, 5.74, and 5.55 d.

Table I. *Effect of Light Quality in Different Systems*

Quotient of blue and red light PFD required for HMR.

Species	Exp. material	b/r[a]	Epidermis[b]	Position[c]	CO_2[d]	Source
PHOTOSYNTHESIS						
22 dicot and monocot		1.3–1.6				(12)
STOMATAL APERTURE						
Fagus sylvatica	Sun leaves	1.33	ad//ab	n	+	(18)
Zea mays	Leaves	1	ab	i	−	(22)
Zea mays	Leaves	0.5 to 1	ad	i	−	(22)
Fagus sylvatica	Shade leaves	0.66	ad//ab	n	+	(18)
Allium cepa	Leaves	0.36	?	...	+	(13)
Picea sitchensis	Leaves	0.20	ad//ab	...	+	(16)
Vicia faba	Leaves	0.18	ab	i	+	(8)
Commelina communis	Isolated epidermis	0.17	ab	...	−	(19)
Xanthium pennsylvanicum	Leaves	0.16	?	?	+	(13)
Xanthium pennsylvanicum	Leaves	0.12	ad+ab	?	+	(11)
Commelina communis	Isolated epidermis	0.11	ad	...	−	(19)
Pinus sylvestris	Leaves	0.11	ad//ab	...	+	(16)
Xanthium strumarium	Leaves	0.10	ab	i	+	(24)
Pinus sylvestris	Leaves	0.08	ad//ab	...	−	(16)
Hedera helix	Leaves	0.011	ab	n	+	Figure 3
Hedera helix	Leaves	0.003	ab	i	+	Figure 3
Paphiopedilum harrisianum	Isolated epidermis	0.00[e]	ab	...	+	(33)

[a] b/r = ratio between quantum flux densities of blue and red light at HMR. [b] ab = abaxial; ad = adaxial; // = resistences in parallel; + = in series; ? = not stated. [c] n = normal; i = inverted; ? = not stated; ... = not applicable. [d] + = atmospheric concentration; − = much lower concentration. [e] No response to red light was observed.

under separate controls. There is a trend in Table I which suggests that the value of the quotient is smaller in plants from shaded environments. The value calculated with the data presented in this paper for the green portions of *H. helix* leaves is the smallest one measured in whole leaves. The leaves of the juvenile phase of this species have been considered similar to those of genotypic shade plants (2). The light sensitivity of the stomata of *H. helix* to blue light could represent an adaptation to this kind of environment. The possibility of such a role for the blue light dependent system has been suggested by Zeiger and Field (32).

Effect of Drought Stress. The g_l of the white and green portions of *H. helix* variegated leaves did not change simultaneously after the onset of drought stress. The g_l of the white portions decreased earlier and recovered more slowly after rewatering (Fig. 4). In contrast, the ψ_l was similar in the white and green portions of the same leaf (Fig. 5). Therefore the earlier closure of the stomata in the white portions cannot be explained by a faster drop of ψ_l in these after interruption of the water supply. Since there were no indications of differences in ψ_l between white and green portion the earlier stomatal closure (Fig. 4) indicates that the stomata in the white portions started to close at higher ψ_l. A difficulty in the analysis of the experiments under white light is that although the irradiance used was saturating in unstressed plants, it is not known whether this was also true under stress. The difference between the time for recovery under white light and that in either red or blue light is not easily explained with available information. The longer lag observed before the start of recovery of the blue light-dependent system was statistically significant (Fig. 6A). As already discussed direct Chl dependent responses were not apparent in our experiments with unstressed plants, hence red light responses should be indirect; hence the faster recovery after rewatering of the indirect PAR dependent system than of the direct blue light dependent one could explain the frequently reported enhancement of the stomatal sensitivity to CO_2 during and after a period of water stress (14, 21). A higher sensitivity to water deficit of the direct, blue light dependent system (located in the epidermis) would also be consistent with the results reported by Fischer (5): that the after effect of drought stress depended more on the epidermis than on the mesophyll, and also his observation that the sensitivity of stomatal aperture to triazines is enhanced after a period of drought stress.

It has been often suggested that at least part of the effect of drought stress on stomatal opening is mediated by an increase of ABA levels (21). Chloroplasts have been indicated as the site of ABA synthesis (15), but conflicting evidence has also been found (7). The higher sensitivity of the white parts of *H. helix* leaves to water stress raises the question whether the stomatal closure in these tissues is not dependent on ABA or the synthesis of ABA is located in the cytoplasm or in guard cells chloroplasts.

The blue light dependent mechanisms has been suggested to be advantageous in shaded or sun-flecked environments (32). It would be interesting to know whether a higher sensitivity of this system to water deficits might also imply an additional ecological benefit.

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